Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury

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Erythropoietin (EPO) plays an important role in the brain's response to neuronal injury. Systemic administration of recombinant human EPO (rhEPO) protects neurons from injury after middle cerebral artery occlusion, traumatic brain injury, neuroinflammation, and excitotoxicity. Protection is in part mediated by antiapoptotic mechanisms. We conducted parallel studies of rhEPO in a model of transient global retinal ischemia induced by raising intraocular pressure, which is a clinically relevant model for retinal diseases. We observed abundant expression of EPO receptor (EPO-R) throughout the ischemic retina. Neutralization of endogenous EPO with soluble EPO-R exacerbated ischemic injury, which supports a crucial role for an endogenous EPO/EPO-R system in the survival and recovery of neurons after an ischemic insult. Systemic administration of rhEPO before or immediately after retinal ischemia not only reduced histopathological damage but also promoted functional recovery as assessed by electroretinography. Exogenous EPO also significantly diminished terminal deoxynucleotidyltransferase-mediated dUTP end labeling labeling of neurons in the ischemic retina, implying an antiapoptotic mechanism of action. These results further establish EPO as a neuroprotective agent in acute neuronal ischemic injury.

Erythropoietin (EPO) has been viewed traditionally as a hematopoietic cytokine produced by the fetal liver and adult kidney in response to hypoxia. Results of recent studies now support a physiological role for EPO within the central nervous system. The expression of EPO and EPO receptors (EPO-Rs) in the central nervous system and the up-regulation of EPO by hypoxia/ischemia *in vitro* and *in vivo* suggest that this cytokine is an important mediator of the brain's response to injury. Consistent with this hypothesis, pretreatment with exogenous EPO protects cultured neurons from hypoxia (1, 2), glutamate excitotoxicity (3), and growth-factor withdrawal (2). When administered systemically, EPO can cross the blood–brain barrier and reduce neuronal injury in animal models of focal ischemic stroke, traumatic brain injury, inflammation, kainate toxicity, and spinal cord injury (2, 4, 5). EPO rescues neurons from acute injury at least in part by inhibiting apoptosis via activation of specific protein kinase pathways (2) and the recruitment of NF-κB (6).

Prior studies of EPO in different models of brain injury raise the possibility that this cytokine may participate in the recovery of retinal neurons from ischemia. Retinal ischemia is a serious and common clinical problem. It occurs as a result of acute vascular occlusion and leads to visual loss in a number of ocular diseases such as acute glaucoma (7), diabetic retinopathy (8), and hypertensive vascular disease (9). Transient global retinal ischemia, for example, shares many similarities with transient global cerebral ischemia. Both cause selective damage of specific subpopulations of neurons. Pyramidal neurons in the CA-1 zone of the hippocampus are selectively vulnerable to transient cerebral ischemic injury (10, 11). Similarly, neurons in the inner nuclear layer (INL) of the retina show significantly enhanced susceptibility to transient retinal ischemia as compared with outer nuclear layer (ONL) neurons (12, 13). Both types of injury are associated with delayed neuronal death, which arises in part by apoptosis (12, 14–17). Further, many of the same signaling pathways are activated in retinal and cerebral ischemia (18–20). Given the similarities between these two types of neuronal injury, we conducted a parallel study of the EPO/EPO-R system in a model of retinal ischemia-reperfusion injury.

EPO is expressed in the human fetal retina (21), but it is unknown whether its expression persists in the adult and whether it has a physiological role in the retina. Using immunohistochemistry and Western blot studies, we observed that EPO-R is increased significantly in the retina by ischemia, which suggests that the ischemic retina can up-regulate recovery pathways that might be enhanced by the exogenous application of EPO. Indeed, systemically administered recombinant human EPO (rhEPO) pretreatment or posttreatment is associated with both histopathological and functional protection of retinal neurons subjected to ischemic injury. We further show that rhEPO inhibits apoptosis after acute retinal ischemia. The present study provides further evidence for a neuroprotective and antiapoptotic effect of EPO/EPO-R and suggests that up-regulating this cytokine system may enhance recovery from ocular diseases involving acute neuronal injuries.

Materials and Methods

rhEPO (Epoetin alfa, Procrit) was obtained from Ortho Biotech (Raritan, NJ). Soluble EPO-R was purchased from R & D Systems.

Retinal Ischemia. Male Sprague–Dawley rats weighing 150–175 g were anesthetized for 45–60 min with an i.p. injection of ketamine (30 mg/kg) and xylazine (2.5 mg/kg). The anterior chamber of the right eye was cannulated with a 27-gauge needle attached to an infusion line of normal saline and to a manometer. The corneal puncture site was sealed with cyanoacrylate cement. The intraocular pressure was raised to 120 mm Hg for a duration up to 60 min. Throughout the ischemic period, systemic blood pressure was monitored continuously with catheterization of the tail artery. Body temperature was maintained at 37 ± 0.5°C by a heating pad and a rectal thermometer probe. Retinal ischemia was confirmed by observing a whitening of the iris and loss of the red reflex of the retina. After 60 min of ischemia, the needle was withdrawn and the intraocular pressure normalized. Gentamicin

Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; ONL, outer nuclear layer; INL, inner nuclear layer; rhEPO, recombinant human EPO; OLM, outer limiting membrane; ILM, inner limiting membrane; OPL, outer plexiform layer; IPL, inner plexiform layer; ERG, electroretinogram; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end labeling; GCL, ganglion cell layer.

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MEDICAL SCIENCES

ophthalmic ointment was applied topically to the right eye before and after cannulation of the anterior chamber. All procedures involving the animals conformed to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Light Microscopy. The right (experimental) and left (untouched control) globes were enucleated 1 week after ischemia and fixed in Trump's fixative. The globes were sectioned in the vertical meridian, and the inferior portion of the eye wall (retina, choroid, and sclera) was embedded in epoxy resin. Sections (1 μm thick) were stained with 1% toluidine blue. The retinal histoarchitecture was evaluated by light microscopy. The thickness of the retinal layers was determined as follows: (*i*) outer limiting membrane (OLM) to inner limiting membrane (ILM), (*ii*) ONL, (*iii*) outer plexiform layer (OPL), (*iv*) INL, and (*v*) inner plexiform layer (IPL) to ILM. Averages for these measurements taken in four adjacent areas within 1 mm of the optic nerve were calculated. Selection of the same topographic region of the retina for all these measurements is important to reduce regional anatomic variations. All measurements were performed in a blinded fashion.

Electroretinograms (ERGs). Rats were dark-adapted overnight and anesthetized briefly with an i.p. injection of ketamine (30 mg/kg) and an i.m. injection of xylazine (2.5 mg/kg). Pupils were dilated with 1% tropicamide (Alcon, Humacao, Puerto Rico) and cyclomydril (0.2% cyclopentolate HCl and 0.1% phenylephrine HCl, Alcon Laboratories, Fort Worth, TX). Animals were kept at 37 ± 0.5°C with a rectal probe and heating pad during the procedure until completely recovered from anesthesia. A platinum electrode was placed on the topically anesthetized cornea. Teca electrolyte electrode gel (Teca, Pleasantville, NY) was used as a conducting medium for the corneal electrode. A reference electrode was placed by the ipsilateral mastoid and a ground electrode was placed close to the midline of the cephalad dorsum. Light stimuli were generated by a Ganzfield xenon flash-tube light source (ERG-jet, Nicolet) with a 0.75 log-flash intensity ($cd{\cdot}s/m^2$). Full-field white-light stroboscopic flashes lasting 10 μsec were presented at a distance of 15 cm and a rate of 1.0 per sec. Neuroelectric signals were impedance-matched through a unity gain preamplifier and differentially amplified further with appropriate band-pass settings. To improve the signal-to-noise ratio, ERG responses elicited by identical stimuli were averaged online by a computer. Amplified signals (200-msec analysis time, 1–1,500-Hz bandwidth) were stored and analyzed. ERG studies were performed in both the injured and contralateral control eyes before ischemia (baseline), immediately after reperfusion, and after 7 days. ERG analysis consisted of determining both the latency to the b-wave peak from the stimulus onset and amplitude from the trough of the a-wave to the peak of the b-wave. The effects of treatment after the a- and b-waves were assessed by dividing the amplitude measured in the experimental eye by the corresponding value of the control eye. All amplitudes were normalized to baseline values and expressed as a percentage of baseline.

Terminal Deoxynucleotidyltransferase-Mediated dUTP End Labeling (TUNEL). This technique is based on the method described by Gavrielli *et al.* (22) but with several modifications. Cryosections (10 μm thick) were incubated in methanol for 15 min at room temperature, washed in 1× PBS for 5 min, and incubated in a bromodeoxy-UTP/terminal deoxynucleotidyltransferase mixture (Roche Molecular Biochemicals) at 37°C for 1 h in a humidified chamber followed by three rinses in 1× PBS. The sections then were incubated with peroxidase converter (Roche Molecular Biochemicals) for 30 min and visualized with diaminobenzidine (DAB kit, Vector Laboratories). Corresponding positive and negative control sections also were prepared. Fluorescent TUNEL staining was performed by using an *in situ* cell death-detection kit (Roche Molecular Biochemicals).

Immunohistochemistry. Eyes were enucleated at 12 (n = 9), 24 (n = 12), 48 (n = 3), and 72 h (n = 12) after 60 min of ischemia and were fixed in 4% paraformaldehyde for 2 h. After removing the anterior segment of the globe, the eye cups were fixed further in paraformaldehyde overnight and then transferred to 25% sucrose solution for an additional 24 h. Corneas and lens were removed, and the eyes were embedded in OCT compound with 2-methylbutane and dry ice. Cryosections (12 μm thick) were prepared at −20°C, fixed in cold methanol for 15 min, rinsed in 1× PBS for 5 min, and incubated with 10% goat serum for 45 min at room temperature. Primary antibodies to EPO and EPO-R were obtained from Santa Cruz Biotechnology. Incubation with each antibody was performed overnight at 4°C. Sections then were washed with 1× PBS three times and incubated with either fluorescein- or rhodamine-conjugated secondary antibody (anti-rabbit IgG, 1:100) at room temperature for 1 h. To investigate cell types expressing EPO and EPO-R, double labeling with cell-specific markers was performed overnight: anti-HPC for amacrine cells (1:150, mouse IgG1, Sigma), anti-Thy-1 for ganglion cells (1:50, mouse IgG1, PharMingen), anti-protein kinase C for bipolar cells (1:50, mouse IgG2a, PharMingen), anti-rhodopsin for photoreceptor cells (RHO42D, 1:10, mouse IgG, gift of Robert Molday), and anti-glial fibrillary acidic protein for Müller cells (1:50, mouse IgG2b, PharMingen). Sections then were washed with 1× PBS three times and incubated with rhodamine-conjugated secondary antibody at room temperature for 2 h. Sections were mounted with Antifade and analyzed under fluorescent microscopy. Corresponding negative controls were prepared by substitution of the primary antibody with 10% normal goat serum in PBS.

Western Blotting. After death and enucleation, retinas harvested at the same time points as for immunohistochemistry were dissected rapidly out, frozen in liquid nitrogen, and subsequently crushed by using a tissue pulverizer (Beckman) chilled by dry ice. These retinas were solubilized in 9 M urea/4% Nonidet P-40/2% 2-mercaptoethanol, at pH 9.5. Protease-inhibitor mixture (P8340, Sigma) consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin was added to inhibit protease activity. Samples were centrifuged for 10 min at ≈14,000 × g. The supernatant was used for SDS/PAGE, and the pellet was discarded. Protein concentration was determined with a modified Bradford assay by using a kit purchased from Bio-Rad. Gel retention was assessed by staining with Coomassie blue (Pierce). Nonspecific binding was blocked with 5% nonfat dry milk in Tween/Tris-buffered saline (TTBS), and then membranes were incubated overnight at 4°C with antibodies against EPO-R (1:5,000, Santa Cruz Biotechnology) or EPO (1:2,000, Santa Cruz Biotechnology) prepared in 5% nonfat dry milk solution in TTBS. Anti-rabbit horseradish peroxidase-conjugated secondary antibody was applied at 1:20,000. Negative controls were performed without primary antibody. Chemiluminescence was developed by using Super Signal West Pico (Pierce). Protein bands were visualized digitally by using a CCDBIO 16SC imaging system (Hitachi Genetic Systems/MiraiBio, Alameda, CA) and quantitated by densitometry (GENE TOOLS and GENE SNAP software, Hitachi Genetic Systems/MiraiBio).

Drug Administration. rhEPO at 5,000 units/kg or normal saline control was administered i.p. 24 h before and just before the onset of ischemia. In a separate set of experiments, rhEPO was administered immediately after the onset of ischemia. To investigate the effect of endogenous EPO on ischemic retinal neurons,

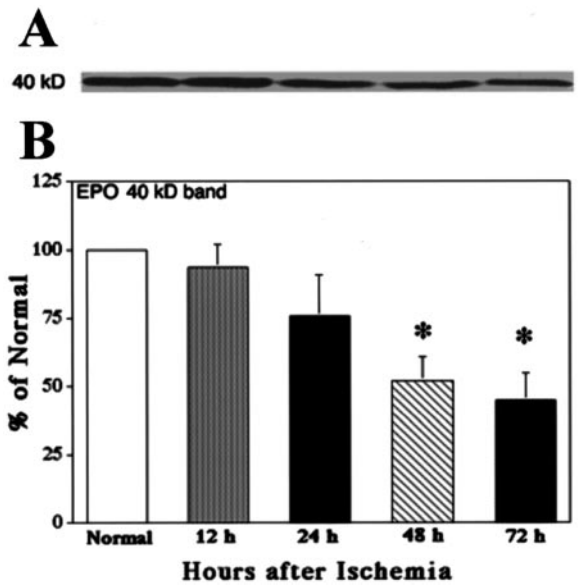

Fig. 1. EPO expression in normal rat retina and retinas after ischemia. (*A*) Time course of EPO protein expression after 60 min of ischemia. EPO is expressed in the normal retina and decreases after ischemia. (*B*) Densitometric analysis demonstrated a significant decrease by 48 h (*, $P < 0.05$). The immunoblots are representative of four experiments.

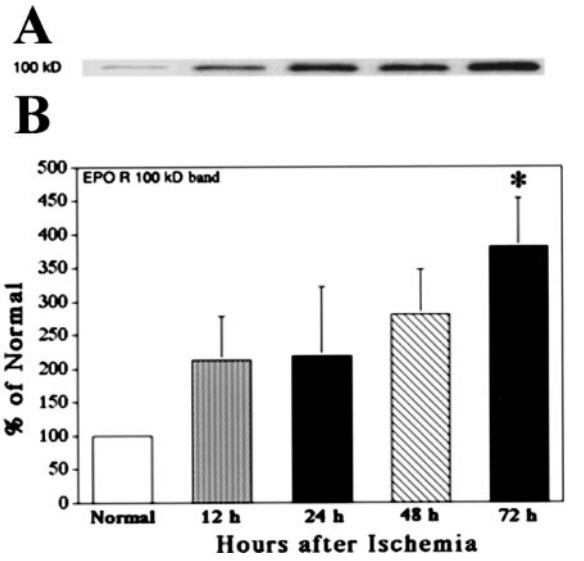

Fig. 2. EPO-R expression in normal rat retina and retinas after ischemia. (*A*) Time course of EPO-R protein expression after 60 min of ischemia. EPO-R was detected in the normal retina and increased after ischemia. (*B*) Densitometric analysis demonstrated a significant increase by 72 h (*, $P < 0.05$). The immunoblots are representative of four experiments.

soluble EPO-R at 2 or 20 ng was given intravitreally just before the induction of ischemia. Control ischemic animals received an intravitreal injection of saline.

Results

EPO and EPO-R Are Expressed in the Ischemic Retina. We first determined the expression of EPO and EPO-R in the normal eye by using specific polyclonal antibodies applied to sections of the rat retina. Immunohistochemistry localized EPO primarily within the inner retina. Double labeling with cell-specific markers identified the majority of EPO-positive cells as amacrine and bipolar neurons (data not shown). Sixty minutes of ischemia led to a dramatic reduction in EPO staining 24 h after reperfusion. By 48 h after ischemia, EPO protein levels dropped to 52% as compared with the normal eye (Fig. 1). Minimal EPO-R protein immunoreactivity was noted in sham-operated control retinas. In contrast, 60 min of ischemia induced pronounced immunoreactivity throughout the retina. Double labeling demonstrated colocalization with ganglion and amacrine neurons and astrocytes (data not shown).

Epo-R protein levels peaked at 72 h and were 4-fold higher than in saline-treated control animals (Fig. 2).

Soluble EPO-R Exacerbates Ischemic Injury. Animals subjected to 30 min of ischemia do not show histopathologic damage but do have a 40% reduction in the ERG b-wave compared with nonischemic controls. This finding made it possible to examine whether the injection of soluble EPO-R exacerbates ischemic damage. Animals were pretreated with either an intravitreal injection of soluble EPO-R (2 or 20 ng) or vehicle. At 7 days of reperfusion, a progressive reduction of the ERG b-wave was observed with increasing concentrations of soluble EPO-R (Fig. 3). The ERG b-wave was reduced significantly compared with vehicle-treated controls ($P < 0.05$). These data provided evidence supportive of an endogenous EPO/EPO-R system that might participate in intrinsic recovery mechanisms after ischemic injury and further suggested that neurons might be rescued by exogenous application of EPO. To test this idea, we administered rhEPO systemically in this retinal ischemia model.

Systemic Administration with rhEPO Is Neuroprotective After Retinal Ischemia. Elevating the intraocular pressure to 120 mm Hg for 45 or 60 min resulted in the typical histopathologic features expected subsequent to acute retinal ischemia with reperfusion (23). In the ischemic eye, along with widespread neuronal degeneration, the entire retinal thickness was reduced compared with the untouched control retinas (Fig. 4). Specifically, there was a 35–40% reduction in the thickness of the ischemic retina (OLM–ILM) as compared with the untouched controls (Table 1), resulting predominantly from marked thinning of the inner retinal layers (INL–ILM). Mild disorganization of the cells in the ONL and of the photoreceptor inner and outer segments was noted also.

Seven days after 45 or 60 min of ischemia, rhEPO-treated eyes showed significant preservation in thickness and histoarchitecture of the inner retina compared with the control groups (Fig. 4*C*; Table 2). rhEPO-treated eyes exhibited functional improve-

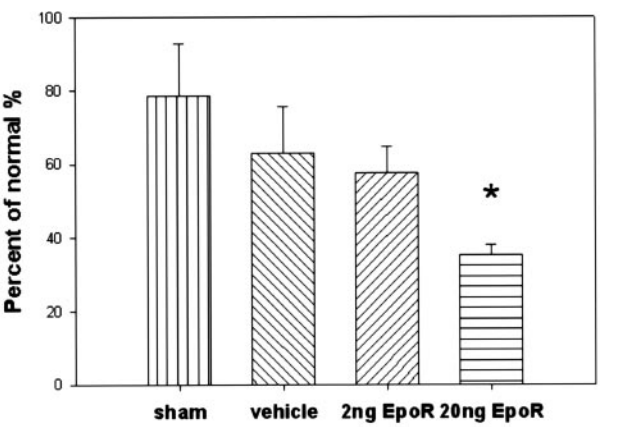

Fig. 3. ERG b-wave measurements of ischemic rat retina at 7 days of reperfusion after 30 min of ischemia. Animals received intravitreal pretreatment with 2 ng of sEPO-R, 20 ng of soluble EPO-R, vehicle, or sham injection before ischemia. Decreased amplitude of the b-wave was observed with increasing concentrations of soluble EPO-R (*, $P < 0.05$).

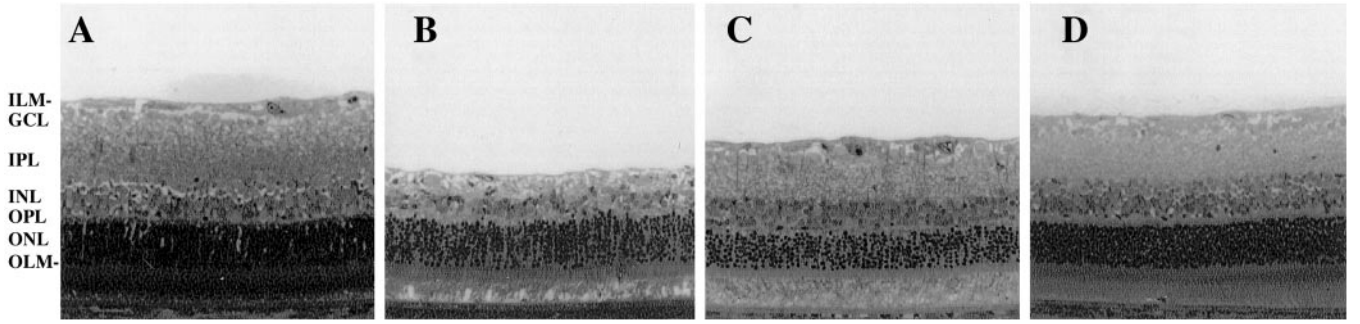

Fig. 4. Representative photomicrographs showing the histological appearances of the nonischemic (control) and ischemic (vehicle and rhEPO-treated) retinas at 7 days of reperfusion after ischemia. Toluidine blue, 1 μm thick; original magnification, ×40. *A*, control; *B*, ischemic (45-min) vehicle-treated; *C*, ischemic (45-min) and EPO-pretreated; *D*, ischemic (45-min), rhEPO-posttreated EPO-treated animals have significantly less retinal thinning compared with vehicle-treated controls (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

ment as evidenced by the preservation of the ERG a- and b-waves in all the treated animals compared with an absent ERG a- and b-wave in all vehicle-treated ischemic controls (Fig. 5 and Table 3).

When rhEPO was administered immediately after 45 min of ischemia, there was marked preservation of the thickness and architecture of the inner retina as well as the ERG a- and b-waves compared with the saline-treated ischemic control groups (Fig. 4*D*). However, rhEPO posttreatment did not alter the histoarchitecture or ERGs in animals exposed to 60 min of ischemia compared with ischemic controls (data not shown).

rhEPO Administration Decreases the Number of TUNEL-Positive Neurons After Retinal Ischemia. No TUNEL-positive cells were seen in the control retinas. However, 24 h after retinal ischemia, TUNEL-positive cells were noted in the vehicle-treated group, predominantly in the ganglion cell layer (GCL) and INL (Table 4). There also were scattered TUNEL-positive cells in the ONL. Pretreatment with rhEPO resulted in significantly fewer TUNEL-positive cells in the GCL and INL compared with the vehicle-treated groups (Table 4), suggesting that rhEPO affords protection by inhibiting apoptosis.

Discussion

Results of the present study further demonstrate the important role of EPO in the survival and recovery of neurons after acute ischemic and reperfusion injury. The normal retina expresses EPO primarily in neurons, and this cytokine was decreased markedly within 48 h after ischemia, which likely is a result of extensive cell loss. In contrast, ischemia induced pronounced up-regulation of EPO-R in a variety of neuronal phenotypes, which suggests the activation of a compensatory response to promote neuronal survival. These findings contrast with prior studies of the brain that have identified astrocytes as the main cellular source of EPO, whereas neurons express EPO-R (3, 21). However, emerging studies have observed that cultured neurons produce EPO (3, 21, 24, 25), and *in vivo* neurons might express EPO after focal cerebral ischemia, but EPO protein levels in one study reportedly have been shown not to change during 1 week after middle cerebral artery occlusion (24). These differences in EPO expression between retinal and cerebral ischemia may be related to the severity of injury but also may depend on different cell types. The temporal induction of EPO-R, on the other hand, is in agreement with prior reports documenting mRNA up-regulation by 12 or 24 h (24, 26) and peak protein levels at 3 days after middle cerebral artery occlusion (24, 26).

In the present study, Müller cells also expressed EPO-R as evidenced by colocalization with glial fibrillary acidic protein staining. Müller cells perform many of the functions carried out by central nervous system astrocytes, oligodendrocytes, and ependymal cells and have been shown to transfer the antioxidant glutathione to neurons under ischemic conditions (27). Müller-cell expression of EPO-R further supports a glial protective response to neuronal injury. A putative protective mechanism by EPO was established further by showing that pretreatment with soluble EPO-R augmented ischemic injury. These findings indicate that the injected soluble EPO-R neutralized endogenous EPO, inhibiting the binding of retinal EPO with neuronal EPO-R. These results are in agreement with prior studies showing that intraventricular infusion of soluble EPO-R potentiates neuronal damage in a model of global cerebral ischemia (28).

Table 1. Retinal layer thickness expressed as percentage of control obtained 7 days after reperfusion

Layer	Duration, min	Pretreatment		Posttreatment	
		Saline	rhEPO	Saline	rhEPO
OLM–ILM	45	71	107***	71	94**
ONL	45	105	113	105	110
OPL	45	97	123	97	102
INL	45	75	119***	75	93*
IPL–ILM	45	48	97***	48	86***
OLM–ILM	60	51	68*	—	—
ONL	60	98	102	—	—
OPL	60	84	86	—	—
INL	60	42	67*	—	—
IPL–ILM	60	28	46*	—	—

Pretreatment corresponds to rhEPO (5,000 units/kg of body weight) or saline administered 24 h before and at the time of reperfusion. Posttreatment corresponds to administration of agent at the time of reperfusion. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ ($n = 10$ for 45-min group, and $n = 5$ for 60-min group).

Table 2. Neuronal counts (percentage of control) obtained from retinas 7 days after reperfusion

Region	Duration	Pretreatment		Posttreatment	
		Saline	rhEPO	Saline	rhEPO
INL	45	71	99***	71	99***
GCL	45	53	93***	53	98***
INL	60	39	70*	—	—
GCL	60	34	54	—	—

*, $P < 0.05$; ***, $P < 0.0005$ ($n = 10$ for 45 min, and $n = 5$ for 60 min).

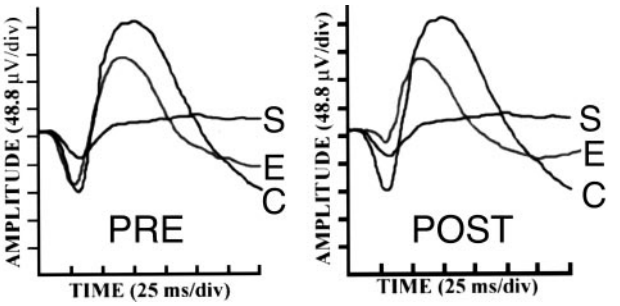

Fig. 5. Representative ERGs obtained 1 week after 45 min of ischemia followed by reperfusion. rhEPO (5,000 units/kg i.p.) given either 24 h before induction of ischemia (PRE) or upon reperfusion (POST) was associated with equivalent protection of neuronal function (see Table 3). C, ERG from control (contralateral) eye; S, saline treatment; E, rhEPO treatment. The a-wave is the small trough at 30 ms, and the b-wave is the peak occurring at 75 ms.

The reduction in EPO expression resulting from ischemic cell damage suggests that this cytokine system is inadequate to withstand an acute neuronal injury. Enhancing EPO-mediated mechanisms of survival may limit neuronal loss in various cerebral and retinal disorders. Exogenous EPO has been shown to reduce ischemic injury in focal and global cerebral ischemic models as well as in traumatic brain injury, neuroinflammation, and kainate excitotoxicity (2, 24, 26, 28). Animals given a single i.p. dose of rhEPO had significant reductions in cortical infarct volume if EPO was given within 6 h after focal ischemia (4). We have corroborated these findings in retinal ischemia by demonstrating that rhEPO not only attenuates retinal damage but also leads to marked recovery of the ERG a- and b-waves after both 45 and 60 min of ischemia. In the 45-min ischemia model, rhEPO extended neuroprotective effects even if administered after the initial injury. Posttreatment, however, did not exert neuroprotection after 60 min of ischemia, a more severe injury. This report demonstrates directly that systemic posttreatment with rhEPO leads to protection of neuronal function. These findings have direct clinical relevance.

Multiple mechanisms may explain how rhEPO protects neurons from acute injury. *In vivo* experiments demonstrate an antiapoptotic role for EPO. Systemic administration of EPO significantly reduces TUNEL-positive cells in the ischemic penumbra after focal cerebral ischemia, suggesting an antiapoptotic effect (2). Similarly, the present study found that ischemic retinas that had received EPO exhibited a marked decrease in TUNEL labeling. Analogous to the well known antiapoptotic action that EPO exerts on erythroid progenitor cells in the bone marrow, EPO may inhibit neuronal apoptosis by up-regulating Bcl-x_L and Bcl-2 (29, 30). EPO also has been shown to inhibit apoptosis of cultured cortical neurons deprived of growth factors or exposed to kainic acid by activating mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKt pathways (2). These signaling mechanisms participate in the antiapoptotic effect of brain-derived neurotrophic factor, which protects retinal neurons from a variety of different injuries (31). EPO therefore may be a particularly effective agent for retinal disorders. EPO also may exert an antiapoptotic effect by recruiting NF-κB (6), which translocates to the nucleus and can activate neuroprotective genes such as superoxide dismutase or inhibitors of apoptosis proteins.

Table 3. ERG a- and b-wave measurements obtained 7 days after reperfusion after ischemia

	Ischemia duration, min	Treatment	Saline	rhEPO
a-wave	45	Pre	65	99
b-wave	45	Pre	11	52
a-wave	45	Post	65	111
b-wave	45	Post	11	60
a-wave	60	Pre	22	60
b-wave	60	Pre	1	22

Data are expressed as percentage of normal baseline. *, $P < 0.05$ ($n = 10$ for 45 min, and $n = 5$ for 60 min).

Table 4. Mean number of TUNEL-positive nuclei per high-power field (×40 objective) of retinal sections 24 h after 45 min of ischemia

Region	Saline	rhEPO
GCL	2.2 ± 0.7	1.0 ± 1.0
INL	33.8 ± 4.6	16.3 ± 10.7*
ONL	12.6 ± 1.5	4.7 ± 2.3*

Treatment administered 24 h and immediately before induction of ischemia. *, $P < 0.05$ compared to control ($n = 5$ each group).

A growing body of evidence also suggests that EPO directly attenuates glutamate excitotoxicity. EPO has been shown to directly inhibit the release of glutamate from neurons (32). Further, pretreatment of cultured neurons leads to dose-dependent reduction in cell death when exposed to glutamate. Simultaneous addition of soluble EPO-R abolishes this protective effect, which argues for an EPO-R-mediated process (3). EPO attenuates neuronal injury after exposure to the glutamate agonist, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (33), and it also protects cultured neurons from hypoxia-ischemia by inhibiting the exocytosis of glutamate (32). These findings are consistent with prior work showing that EPO can reduce neuronal damage induced by kainate toxicity *in vivo* (4). The involvement of excitotoxic and apoptotic mechanisms in ischemic disease raises the importance of designing agents that target multiple cell death pathways. Up-regulating EPO-R-mediated signaling therefore may be a more rational therapeutic approach for neuronal disorders in which both pathophysiologic processes play a role.

The results of this study and others suggest that the systemic administration of rhEPO could augment recovery pathways, promoting neuronal viability and restoring neuronal function after an acute injury. Our findings also demonstrate that agents that inhibit apoptosis can preserve neuronal function after acute ischemic injury. rhEPO may represent a therapeutic agent for several retinal diseases including acute glaucoma, acute retinal vascular occlusion, diabetic retinopathy, and hypertensive vascular disease. The erythropoietic effects, however, may likely limit the clinical utility of EPO for chronic ischemic diseases such as diabetic and hypertensive retinopathy. Such limitations raise the importance of further elucidating those signaling pathways mediating erythropoiesis versus neuroprotection. Future work using agents that selectively promote neuroprotective rather than erythropoietic effects may be enlightening.

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